A harvested sprout according to the present invention can be incorporated immediately into food products such as fresh salads, sandwiches or drinks. Alternatively, the growth of the harvested sprout can be arrested by some active human intervention, for example by refrigeration, at a stage of growth prior to the 2-leaf stage, typically between 1 and 14 days after germination of seeds. Growth arrest can also be accomplished by removing a sprout from its substrate and/or water source. Freezing, drying, baking, cooking, lyophilizing and boiling are among the many treatments that can be used to arrest growth. These may also be useful for either preserving myrosinase activity in the sprout (*e.g.*, lyophilizing) or for inactivating myrosinase activity in the sprout (*e.g.*, boiling), as is desired in a particular application.

The harvested sprout can also be allowed to mature further, under different growing conditions, prior to incorporation into a food product. For example, the sprout can be harvested at a very young age of development, such as 1 to 2 days after seed imbibition. The sprout can then be allowed to mature under different growing conditions, such as increased or decreased light intensity, temperature or humidity; exposure to ultraviolet light or other stresses; or addition of exogenous nutrients or plant growth regulators (hormones). The sprout is then immediately incorporated into a food product, such as for fresh consumption in salads. Alternatively, the growth of the sprout is arrested and/or further treated by means of lyophilization, drying, extracting with water or other solvents, freezing, baking, cooking, or boiling, among others.

A sprout is suitable for human consumption if it does not have non-edible substrate such as soil attached or clinging to it. Typically the sprouts are grown on a non-nutritive solid support, such as agar, paper towel,

Exhibit 1-B(ii)

blotting paper, Vermiculite, Perlite, etc., with water and light supplied. Thus, if a sprout is not grown in soil, but on a solid support, it does not need to be washed to remove non-edible soil. If a sprout is grown in a particulate solid support, such as soil, Vermiculite, or Perlite, washing may be required to achieve a sprout suitable for human consumption.

Sprouts can be grown in containers which are suitable for shipping and marketing. Typically such containers are plastic boxes or jars which contain a wetted pad at the bottom. The containers allow light to penetrate while providing a mechanically protective barrier. Numerous methods for the cultivation of sprouts are known, as exemplified by U.S. Patent Nos. 3,733,745, 3,643,376, 3,945,148, 4,130,964, 4,292,760 or 4,086,725. Food products containing the sprouts of the instant invention can be stored and shipped in diverse types of containers such as jars, bags and boxes, among many others.

Sprouts suitable as sources of cancer chemoprotectants are generally cruciferous sprouts, with the exception of cabbage (*Brassica oleracea capitata*), cress (*Lepidiumsativum*), mustard (*Sinapis alba* and *S. niger*) and radish (*Raphanus sativus*) sprouts. The selected sprouts are typically from the family *Cruciferae*, of the tribe *Brassiceae*, and of the subtribe *Brassicinae*. Preferably the sprouts are *Brassica oleracea* selected from the group of varieties consisting of *acephala* (kale, collards, wild cabbage, curly kale), medullosa (marrowstem kale), ramosa (thousand head kale), *alboglabra* (Chinese kale), *botrytis* (cauliflower, sprouting broccoli), *costata* (Portuguese kale), *gemmifera* (Brussels sprouts), *gongylodes* (kohlrabi), *italica* (broccoli), *palmifolia* (Jersey kale), *sabauda* (savoy cabbage), *sabellica* (collards), and *selensia* (borecole), among others.

Particularly useful broccoli cultivars to be used in the claimed method are Saga, DeCicco, Everest, Emerald City, Packman, Corvet, Dandy Early, Emperor, Mariner, Green Comet, Green Valiant, Arcadia, Calabrese Caravel, Chancellor, Citation, Cruiser, Early Purple Sprouting Red Arrow, Eureka, Excelsior, Galleon, Ginga, Goliath, Green Duke, Greenbelt, Italian Sprouting, Late Purple Sprouting, Late Winter Sprouting White Star, Legend, Leprechaun, Marathon, Mariner, Minaret (Romanesco), Paragon, Patriot, Premium Crop, Rapine (Spring Raab), Rosalind, Salade (Fall Raab), Samurai, Shogun, Sprinter, Sultan, Taiko, and Trixie. However, many other broccoli cultivars are suitable.

Particularly useful cauliflower cultivars are Alverda, Amazing, Andes, Burgundy Queen, Candid Charm, Cashmere, Christmas White, Dominant, Elby, Extra Early Snowball, Fremont, Incline, Milkyway Minuteman, Rushmore, S-207, Serrano, Sierra Nevada, Siria, Snow Crown, Snow Flake, Snow Grace, Snowbred, Solide, Taipan, Violet Queen, White Baron, White Bishop, White Contessa, White Corona, White Dove, White Flash, White Fox, White Knight, White Light, White Queen, White Rock, White Sails, White Summer, White Top, Yukon. However, many other cauliflower cultivars are suitable.

Suitable sprouts will have at least 200,000 units per gram of fresh weight of Phase 2 enzyme-inducing potential following 3-days incubation of seeds under conditions in which the seeds germinate and grow. Preferably the sprouts will have at least 250,000 units of inducer potential per gram of fresh weight, or even 300,000 units, 350,000 units, 400,000 units, or 450,000 units. Some samples have been found to contain greater than 500,000 units per gram of fresh weight at 3-days of growth from seeds.

The level of inducing activity and inducing potential has been found to vary among crucifers and even among

cultivars. Most preferably, the sprouts are substantially free of indole glucosinolates and their breakdown products which have Phase 1 enzyme-inducing potential in mammalian cells, and substantially free of toxic levels of goitrogenic nitriles and glucosinolates such as hydroxybutenyl glucosinolates, which upon hydrolysis yield oxazolidonethiones which are goitrogenic. Mature Brussels sprouts and rapeseed are rich in these undesirable glucosinolates.

Non-toxic solvent extracts according to the invention are useful as healthful infusions or soups. Non-toxic or easily removable solvents useful for extraction according to the present invention include water, liquid carbon dioxide or ethanol, among others. The sprouts can be extracted with cold, warm, or preferably hot or boiling water which denature or inactivate myrosinase. The residue of the sprouts, post-extraction, may or may not be removed from the extract. The extraction procedure may be used to inactivate myrosinase present in the sprouts. This may contribute to the stability of the inducer potential. The extract can be ingested directly, or can be further treated. It can, for example, be evaporated to yield a dried extracted product. It can be cooled, frozen, or freeze-dried. It can be mixed with a crucifer vegetable which contains an active myrosinase enzyme. This will accomplish a rapid conversion of the glucosinolates to isothiocyanates, prior to ingestion. Suitable vegetables that contain active myrosinase are of the genus *Raphanus*, especially daikon, a type of radish.

Seeds, as well as sprouts have been found to be extremely rich in inducer potential. Thus it is within the scope of the invention to use crucifer seeds in food products. Suitable crucifer seeds may be ground into a flour or meal for use as a food or drink supplement. The flour or meal is incorporated into breads, other baked goods, or health drinks or shakes. Alternatively, the seeds may be extracted with a non-toxic solvent such as

water, liquid carbon dioxide or ethanol to prepare soups, teas or other drinks and infusions. The seeds can also be incorporated into a food product without grinding. The seeds can be used in many different foods such as salads, granolas, breads and other baked goods, among others.

Food products of the instant invention may include sprouts, seeds or extracts of sprouts or seeds taken from one or more different crucifer genera, species, varieties, subvarieties or cultivars. It has been found that genetically distinct crucifers produce chemically distinct Phase 2 enzyme-inducers. Different Phase 2 enzyme-inducers detoxify chemically distinct carcinogens at different rates. Accordingly, food products composed of genetically distinct crucifer sprouts or seeds, or extracts or preparations made from these sprouts or seeds, will detoxify a broader range of carcinogens.

Glucosinolates and/or isothiocyanates can be purified from seed or plant extracts by methods well known in the art. *See* Fenwick *et al.*, *CRC Crit. Rez. Food Sci. Nutr.* 18: 123-201 (1983) and Zhang *et al.*, *Pro. Natl Acad. Sci. USA* 89: 2399-2403 (1992). Purified or partially purified glucosinolate(s) or isothiocyanate(s) can be added to food products as a supplement. The dose of glucosinolate and/or isothiocyanate added to the food product preferably is in the range of 1 $\mu$mol to 1,000 $\mu$mols. However, the dose of glucosinolate and/or isothiocyanate supplementing the food product can be higher.

The selection of plants having high Phase 2 enzyme-inducer potential in sprouts, seeds or other plant parts can be incorporated into *Cruciferae* breeding programs. In addition, these same breeding programs can include the identification and selection of cultivars that produce specific Phase 2 enzyme-inducers, or a particular spectrum of Phase 2 enzyme-inducers. Strategies for the crossing, selection and breeding of new cultivars of

*Cruciferae* are well known to the skilled artisan in this field. *Brassica Crops and Wild Allies: Biology & Breeding*; S. Tsunoda *et al.* (eds), Japan Scientific Societies Press, Tokyo pp. 354 (1980). Progeny plants are screened for Phase 2 inducer activity or the chemical identity of specific Phase 2 enzyme-inducers produced at specific plant developmental stages. Plants carrying the trait of interest are identified and the characteristic intensified or combined with other important agronomic characteristics using breeding techniques well known in the art of plant breeding.

### *Example 1*

### *COMPARISON OF CRUCIFEROUS SPROUT INDUCING POTENTIAL*

Sprouts were prepared by first surface sterilizing seeds of different species from the cruciferae family with a 1 min treatment in 70% ethanol, followed by 15 min in 1.3% sodium hypochlorite containing approximately 0.001% Alconox detergent. Seeds were grown in sterile plastic containers at a density of approximately 8 seeds/$cm^2$ for from 1 to 9 days on a 0.7% agar support that did not contain added nutrients. The environment was carefully controlled with broad spectrum fluorescent lighting, humidity and temperature control. The seeds and sprouts were incubated under a daily cycle of 16 hours light at 25°C and 8 hours dark at 20°C.

Sprouts were harvested following 3-days of incubation and immediately plunged into 10 volumes of a mixture of equal volumes of DMF/ACN/DMSO at -50°C. This solvent mixture has a freezing point of approximately -33°C, but when admixed with 10% water, as found in plant material, the freezing point is depressed to below -64°C. The actual freezing point depression is even greater with plant material.

Homogenization was accomplished either by manually grinding the samples in a glass-on-glass homogenizer in

the presence of a small amount of the total solvent used, then gradually adding more solvent or homogenizing the sample in 10 volumes of solvent using a Brinkman Polytron Homogenizer for 1 min at half-maximum power. The homogenate was then centrifuged to remove remaining particulates and stored at -20°C until assayed.

Inducer potential of plant extracts prepared as described above, was determined by the microtiter plate bioassay method as described in the Definitions section above.

Broccoli and cauliflower sprouts harvested and assayed at 3-days after incubation of seeds under growth conditions have Phase 2 enzyme-inducer potential greater than 200,000 units/g fresh weight. On the other hand, cabbage, radish, mustard and cress have Phase 2 enzyme-inducer potential of less than 200,000 units/g fresh weight when assayed at the same time point.

*Example 2*

*VARIATION IN INDUCER POTENTIAL AMONG DIFFERENT BROCCOLI CULTIVARS*

There is variation in inducer potential among different broccoli cultivars. In addition, most of the inducer potential in crucifers is present as precursor glucosinolates. The inducer activity and inducer potential of market stage broccoli heads was determined following DMF/ACN/DMSO extractions and assay of QR activity as described above.

Bioassay of homogenates of such market stage broccoli heads, with and without the addition of purified plant myrosinase, showed that the amount of QR activity found in the absence of myrosinase was less than 5% of that observed with added myrosinase. These observations confirmed previous suggestions (see Matile *et al.*,

*Biochem. Physiol. Pflanzen* 179: 5-12 (1984)) that uninjured plants contain almost no free isothiocyanates.

TABLE 1
Effect of Myrosinase on Inducer Activity
of Market-Stage Broccoli Plant Heads

| Broccoli cultivar | Units per gram (wet weight) vegetable | |
|---|---|---|
| | -myrosinase | +myrosinase |
| DeCicco | 5,882 | 37,037 |
| Calabrese Corvet | 1,250 | 41,666 |
| Everest | * | 8,333 |
| Dandy Early | * | 20,000 |
| Emperor | * | 13,333 |
| Saga | 5,000 | 13,333 |
| Emerald City | * | 12,500 |

* Below limits of detection (833 units/g).

As can be observed in Table 1, most of the plant inducer potential is derived from glucosinolates following hydrolysis by myrosinase to form isothiocyanates. Hence, hydrolysis is required for biological activity.

### *Example 3*
### *INDUCER POTENTIAL IS HIGHEST IN SEEDS AND DECREASES AS SPROUTS MATURE*

Phase 2 enzyme-inducer potential is highest in seeds and decrease gradually during early growth of seedlings. Plants were prepared by first surface sterilizing seeds of *Brassica oleracea* variety *italica* cultivars Saga and DeCicco with a 1 min treatment in 70% ethanol, followed

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by 15 min in 1.3% sodium hypochlorite containing approximately 0.001% Alconox detergent. Seeds were grown in sterile plastic containers at a density of approximately 8 seeds/cm$^2$ on a 0.7% agar support that did not contain added nutrients. The environment was carefully controlled with broad spectrum fluorescent lighting, humidity and temperature control. The seeds and sprouts were incubated under a daily cycle of 16 hours light at 25°C and 8 hours dark at 20°C.

Each day plants were rapidly and gently collected from the surface of the agar from replicate containers. The plants were harvested gently to minimize glucosinolate hydrolysis by endogenous myrosinase released upon plant wounding. Samples containing approximately 40 sprouts were homogenized in 10 volumes of DMF/ACN/DMSO solvent at -50°C which dissolves nearly all the non-lignocellulosic plant material.

Harvested plants were homogenized and QR activity with and without myrosinase, was determined as described above. As can be seen in Figure 1, Phase 2 enzyme-inducer potential per gram of plant is highest in seeds, but decreases gradually following germination. No detectable (less than 1000 units/g) QR inducer activity was present in the absence of added myrosinase.

*Example 4*

*SPROUTS HAVE HIGHER INDUCER POTENTIAL THAN MARKET STAGE PLANTS*

The cruciferous sprouts of the instant invention have higher Phase 2 enzyme-inducer potential than market stage plants. More specifically, sprouts have at least a 5-fold greater Phase 2 enzyme-inducing potential than mature vegetables. For example, total inducing potential of 7-day-old broccoli sprouts, extracted with DMF/ACN/DMSO and treated with myrosinase, as described

above, were 238,000 and 91,000 units/g fresh weight, compared to 25,000 and 20,000 units/g fresh weight for field-grown heads of broccoli cultivars Saga and DeCicco, respectively.

Sprout extracts of over 40 different members of the *Cruciferae* have now been bioassayed and broccoli sprouts remain the most Phase 2 enzyme-inducer-rich plants tested. Total inducing potential of organic solvent extracts of market stage and sprout stage broccoli and daikon is shown in Table 2.

TABLE 2

Comparison of Inducer Potential in Sprouts and Mature Vegetables

| Vegetable Cultivar* | Activity (units/g fresh weight) Mature Vegetable | Sprout** | -Fold Difference |
|---|---|---|---|
| **DAIKON** | | | |
| Miura | 625 | 26,316 | 42 |
| Tenshun | 3,333 | 33,333 | 10 |
| Hakkai | 1,471 | 16,667 | 11 |
| Ohkura | 2,857 | 50,000 | 18 |
| **BROCCOLI** | | | |
| Saga | 25,000 | 476,000 | 19 |
| DeCicco | 25,000 | 625,000 | 25 |
| Everest | 8,333 | 1,087,000 | 130 |
| Emerald City | 12,500 | 833,000 | 67 |
| Packman | 20,000 | 556,000 | 28 |

*The commercial portion of each plant was sampled (*e.g.* the taproot of *Raphanus sativus* variety *radicola* [radish]), and heads of *Brassicsa oleracea* variety *italica* [broccoli]). Myrosinase was added to all extracts tested.

**Broccoli sprouts were 1-day old and daikon seedlings were 4-5-days old.

Sprouts of the broccoli cultivar Everest contained 130-fold more inducer potential (units/g fresh weight) than mature vegetables. The inducer activity in broccoli was significantly higher than in daikon.

### *Example 5*

### *INDUCER POTENTIAL OF BROCCOLI SPROUT EXTRACTS*

Inducer potential of a series of water extracts of 3-day old broccoli sprouts of the cultivar Saga were determined. Plants were prepared by first surface sterilizing seeds of *Brassica oleracea* variety *italica* (broccoli) cultivar Saga by a 1 min treatment in 70% ethanol, followed by 15 min in 1.3% sodium hypochlorite containing approximately 0.001% Alconox detergent. Seeds were grown in sterile plastic containers at a density of approximately 8 seeds/cm$^2$ for 72 hours on a 0.7% agar support that did not contain added nutrients. The environment was carefully controlled with broad spectrum fluorescent lighting, humidity and temperature control (16 hours light, 25°C / 8 hours dark, 20°C).

Plants were rapidly and gently collected from the surface of the agar to minimize glucosinolate hydrolysis by endogenous myrosinase released upon plant wounding. Sprouts (approximately 25 mg fresh wt/sprout) were gently harvested and immediately and rapidly plunged into approximately 3 volumes of boiling water in order to inactivate endogenous myrosinase as well as to extract glucosinolates and isothiocyanates from the plant tissue. Water was returned to a boil and maintained at a rolling boil for 3 min. The sprouts were then either strained from the boiled infusion (tea soup) ~~(tea, soup)~~ or homogenized in it, and the residue then removed by filtration or centrifugation.

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Data in Table 3 represent both homogenates and infusions. Preparations were stored at -20°C until assayed. Inducer potential of plant extracts, prepared

as described above, was determined as described in Definitions section above.

TABLE 3
Inducer Potentials of Hot Water Extracts of 3-Day Saga Broccoli Sprouts

| EXTRACT NO. | units/g fresh weight |
|---|---|
| 1 | 500,000 |
| 2 | 370,000 |
| 3 | 455,000 |
| 4 | 333,000 |
| 5 | 435,000 |
| 6 | 333,000 |
| 7 | 625,000 |
| 8 | 250,000 |
| 9 | 313,000 |
| 10 | 357,000 |
| 11 | 370,000 |
| 12 | 370,000 |
| 13 | 217,000 |
| 14 | 222,000 |
| 15 | 1,000,000 |
| 16 | 714,000 |
| 17 | 435,000 |
| 18 | 1,250,000 |
| 19 | 263,000 |
| AVERAGE | 464,000 ± 61,600 S.E.M. |

Some variability in the amount of Phase 2 enzyme-inducer potential was detected. High levels of Phase 2 enzyme-inducer potential, however, were consistently observed.

### *Example 6*
### *HOT WATER BROCCOLI EXTRACTS TREATED WITH DAIKON MYROSINASE*

QR activity in a hot water broccoli extract increased in the presence of a vegetable source of myrosinase. An aqueous extraction of 3-day old sprouts of broccoli cultivar Saga grown on water agar, in which myrosinase was inactivated by boiling for 3 min, was divided into 6 different 150 ml aliquots. Nine-day old daikon sprouts, a rich source of the enzyme myrosinase, were added to this cooled infusion in amounts equivalent to 0, 5, 9, 17, 29 and 40% (w/w) of the broccoli. QR activity, as determined in the Definition section, of the control extracts containing 0% daikon was 26,300 units/gram fresh weight while QR activity of the extracts that had received daikon as a source of myrosinase ranged from 500,000 to 833,000 units/gram fresh weight of broccoli. Accordingly, myrosinase present in the daikon sprouts, increased the QR activity in the broccoli extract greater than 19-fold.

### *Example 7*
### *GLUCORAPHANIN AND GLUCOERUCIN ARE THE PREDOMINANT GLUCOSINOLATES IN HOT WATER EXTRACTS OF BROCCOLI (CULTIVAR SAGA) SPROUTS*

*Paired Ion Chromatography (PIC).* Centrifuged hot water extracts of 3-day-old broccoli (cultivar Saga) sprouts were subjected to analytical and preparative PIC on a reverse phase C18 Partisil ODS-2 HPLC column in ACN/$H_2O$ (1/1, by vol.) with tetraoctylammonium (TOA) bromide as the counter-ion. Only three well-separated peaks were detected: peak A eluted at 5.5 min, B at 11.5

min, and C at 13 min at a molar ratio (A:B:C) ~~[A:B:C]~~ of ca. 2.5 : 1.6 : 1.0 (monitored by UV absorption at 235 nm), and they disappeared if the initial extracts were first treated with highly purified myrosinase. Peaks A, B, and C contained no significant inducer activity, and cyclocondensation assay of myrosinase hydrolysates showed that only Peaks A and C produced significant quantities of isothiocyanates, accounting for all the inducer activity. See Zhang *et al.*, *Anal. Biochem.* 205: 100-107 (1992). Peak B was not further characterized. Peaks A and C were eluted from HPLC as TOA salts but required conversion to ammonium salts for successful mass spectroscopy, NMR and bioassay. The pure peak materials were dried in a vacuum centrifuge, redissolved in aqueous 20 mM $NH_4Cl$, and extracted with chloroform to remove excess TOA bromide. The ammonium salts of glucosinolates remained in the aqueous phase, which was then evaporated.

*Identification of Glucosinolates.* The ammonium salts of Peaks A and C were characterized by mass spectrometric and NMR techniques: (a) negative ion Fast Atom Bombardment (FAB) on a thioglyerol matrix; this gave values of 436 (Peak A) and 420 (Peak C) amu for the negative molecular ions, and (b) high resolution NMR, as shown in Figure 2, provided unequivocal identification of the structure. Peak A is glucoraphanin (4-methylsulfinylbutyl glucosinolate), and Peak C is the closely related glucoerucin (4-methythiobutyl glucosinolate). These identifications and purity are also consistent with the inducer potencies; Peaks A and C, after myrosinase hydrolysis had potencies of 36,100 and 4,360 units/$\mu$mol, respectively, compared with reported CD values of 0.2 $\mu$M (33,333 units/$\mu$mol) for sulforaphane and 2.3 $\mu$M (2,900 units/$\mu$mol) for erucin. CD values are the concentrations of a compound required to double the QR specific activity in Hepa 1c1c7 murine hepatoma cells. Since there are no other glucosinolate peaks, and the inducer activity of peak A and C account for the total inducer activity of the extracts, it is

therefore likely that in this cultivar of broccoli, there are no significant quantities of other inducers, *i.e.,* no indole or hydroxyalkenyl glucosinolates. Further, the isolated compounds are therefore substantially pure.

### *Example 8*

### *COMPARISON OF AQUEOUS AND ORGANIC SOLVENT TECHNIQUES FOR EXTRACTION OF INDUCER POTENTIAL*

Plants were prepared by first surface sterilizing seeds of *Brassica oleracea* variety *italica* (broccoli) cultivar Saga, with 70% ethanol followed by 1.3% sodium hypochlorite and 0.001% alconox. The seeds were grown in sterile plastic containers at a density of approximately 8 seeds/cm$^2$ for 72 hours on a 0.7% agar support that did not contain added nutrients. The environment was carefully controlled with broad spectrum fluorescent lighting, humidity, and temperature control (16 hours light, 25°C/8 hours dark, 20°C).

The plants were rapidly and gently collected from the surface of the agar to minimize glucosinolate hydrolysis by endogenous myrosinase released upon plant wounding. A portion of the plants was homogenized with 10 volumes of the DMF/ACN/DMSO solvent at -50°C, as described in Example 1, which dissolves nearly all the non-lignocellulosic plant material. Alternatively, the bulk of the harvested plants was plunged into 5 volumes of boiling water for 3 min to inactivate endogenous myrosinase and to extract glucosinolates and isothiocyanates. The cooled mixture was homogenized, centrifuged, and the supernant fluid was stored at -20°C.

Inducer potential of plant extracts, prepared by the two methods described above, was determined by the microtiter plate bioassay as described above. Typical inducer potentials in an average of 5 preparations were 702,000 (DMF/ACN/DMSO extracts) and 505,000 (aqueous extracts) units/g fresh weight of sprouts.

Spectrophotometric quantitation of the cyclocondensation product of the reaction of isothiocyanates with 1,2-benzenedithiole was carried out as described in Zhang *et al.*, *Anal. Biochem.* 205: 100-107 (1992). Glucosinolates were rapidly converted to isothiocyanates after addition of myrosinase. About 6% of the total hot water extractable material [dissolved solids] consisted of glucosinolates. These results demonstrate that (a) isothiocyanate levels in the crude plant extracts are extremely low; (b) myrosinase rapidly converts abundant glucosinolates to isothiocyanates; (c) hot water extraction releases over 70% of the inducer activity extractable with a triple solvent mixture permitting recovery of most of the biological activity in a preparation that is safe for human consumption; and (d) over 95% of the inducing potential in the intact plant is present as glucosinolates and therefore no other inducers are present in biologically significant quantities.

### *Example 9*

### *DEVELOPMENTAL REGULATION OF GLUCOSINOLATE PRODUCTION*

Preliminary experiments in which field grown broccoli (cultivar DeCicco) was harvested at sequential time points from the same field indicated that on a fresh weight basis, inducer potential declined from the early vegetative stage through commercial harvest, but appeared to increase at late harvest (onset of flowering). These data suggested that inducer potential might be highest in seeds. Subsequent studies have shown that when seeds of 8 broccoli cultivars were surface sterilized and grown under gnotobiotic conditions, Phase 2 enzyme-inducer potential was highest in seeds and declined progressively (on a fresh weight basis) over time throughout the first 14 days of seedling growth.

Expressed on a per plant basis, however, activity remained constant over this period, suggesting that at

this early stage of growth there was no net synthesis of glucosinolates. However, when the glucosinolate profiles of market stage broccoli heads and 3 day old sprouts (cultivar Emperor) were compared, there was a profound difference in the apparent glucosinolate compositions of these plants.

Sprouts were prepared by first surface sterilizing seeds of *Brassica oleracea* variety *italica* (broccoli) cultivar Emperor with a 1 minute treatment in 70% ethanol, followed by 15 min in 1.3% sodium hypochlorite with approximately 0.001% Alconox detergent. Seeds were grown in sterile plastic containers at a density of approximately 8 seeds/$cm^2$ for 72 hours on a 0.7% agar support that did not contain added nutrients. The environment was carefully controlled; broad spectrum fluorescent lighting, humidity and temperature control (16 hours light, 25°C / 8 hours dark, 20°C).

Plants were rapidly and gently collected from the surface of the agar to minimize glucosinolate hydrolysis by endogenous myrosinase released upon plant wounding. Sprouts (approximately 25 mg fresh wt/sprout), were gently harvested and immediately and rapidly plunged into approximately 3 volumes of boiling water in order to inactivate endogenous myrosinase as well as to extract glucosinolates and isothiocyanates from the plant tissue. Water was returned to a boil and maintained at a rolling boil for 3 min. The sprouts were then strained from the boiled infusion (tea, soup) and the infusion was stored at -20°C until assayed.

Market stage heads were obtained by germinating seeds of the same seedlot in a greenhouse in potting soil, transplanting to an organically managed field in Garrett County, MD and harvested at market stage. Heads were immediately frozen upon harvest, transported to the laboratory on ice and extracts were prepared in an identical fashion to those described above for sprouts

except that approximately 3 gram floret tissue samples were used for extraction.

Inducer potential of plant extracts, prepared as described above, was determined by the microtiter plate bioassay method as described in Example 1. Paired ion chromatography revealed two major peaks, probably glucobrassicin and neo-glucobrassicin, in extracts of market stage heads with similar retention times to glucobrassicin (indole-3-ylmethyl glucosinolate) and neo-glucobrassicin (1-methoxyindole-3-ylmethyl glucosinolate). This observation is consistent with published reports on the glucosinolate composition of mature broccoli plants. However, paired ion chromatography under the same conditions of identically prepared extracts of 3-day-old sprouts showed absence of glucobrassicin or neo-glucobrassicin. Additionally, 3-day-old sprouts of different broccoli cultivars produce different mixtures of glucosinolates. Accordingly, glucosinolate production is developmentally regulated.

***Example 10***

***EVALUATION OF ANTICARCINOGENIC ACTIVITIES OF BROCCOLI SPROUT PREPARATIONS IN THE HUGGINS DMBA (9,10 DIMETHYL-1,2-BENZANTHRACENE) MAMMARY TUMOR MODEL***

Sprouts were prepared by first surface sterilizing seeds of *Brassica oleracea* variety *italica* (broccoli) cultivar Saga with a 1 min treatment in 70% ethanol, followed by 15 min in 1.3% sodium hypochlorite with approximately 0.001% Alconox detergent. Seeds were grown in sterile plastic containers at a density of approximately 8 seeds/cm$^2$ for 72 hours on a 0.7% agar support that did not contain added nutrients. The environment was carefully controlled with broad spectrum fluorescent lighting, humidity and temperature control (16 hours light, 25°C / 8 hours dark, 20°C).

The plants were rapidly and gently collected from the surface of the agar to minimize glucosinolate hydrolysis by endogenous myrosinase released upon plant wounding. A large quantity of sprouts was harvested by immediately and rapidly plunging into approximately 3 volumes of boiling water in order to inactivate endogenous myrosinase, as well as extracting glucosinolates and isothiocyanates from the plant tissue. Water was returned to a boil and maintained at a rolling boil for 3 min. Sprouts were then strained from the boiled infusion (tea, soup) and the infusion was lyophilized and stored as a dry powder at -20°C (designated Prep A). Other sprouts, similarly prepared were extracted with boiling water, cooled to 25°C and were amended with a quantity of 7 day old daikon sprouts equivalent to approximately 0.5% of the original fresh weight of broccoli sprouts. This mixture was homogenized using a Brinkman Polytron Homogenizer and incubated at 37°C for 2 hours following which it was filtered through a sintered glass filter, lyophilized as above and stored as a dried powder at -20°C (designated Prep B).

QR inducer activity and inducer potential of plant extracts, prepared as described above, was determined by the microtiter plate bioassay method as described above. The induction of QR activity in preparation A is largely due to glucosinolates; predominantly glucoraphanin, which is the glucosinolate of sulforaphane, but this preparation also contains some glucoerucin, which is the sulfide analog of glucoraphanin. The induction QR activity of preparation B is almost exclusively due to isothiocyanates arising from treatment of glucosinolates with myrosinase.

Female Sprague-Dawley rats received at 35 days of age were randomized; 4 animals per plastic cage. All animals received 10 mg DMBA, by gavage in 1 ml sesame oil, at age 50 days. Sprout preparations (A or B) or vehicle control were given by gavage at 3, 2 & 1 day prior to DMBA, on

the day of DMBA (2 hr prior to the DMBA dose) and on the day following DMBA dosing. The vehicle used was 50% Emulphor 620P / 50% water. Animals were maintained on a semi-purified AIN-76A diet *ad libitum* from the time of receipt until termination of the experiment (167 days of age).

*TABLE 4*

*ANTICARCINOGENIC ACTIVITIES OF BROCCOLI SPROUT EXTRACTS IN THE DMBA RAT MAMMARY TUMOR MODEL*

| GROUP | TREATMENT | NUMBER OF ANIMALS AT TERMINATION | TOTAL TUMOR NUMBER | MULTIPLICITY: NUMBER OF TUMORS PER RAT |
|---|---|---|---|---|
| CONTROL | DMBA only | 19 | 34 | 1.79 |
| PREPARATION A (Glucosinolate) | 324 mg/dose (100 μmol sulforaphane equiv.) | 18 | 19 | 1.05 |
| PREPARATION B (Isothiocyanate) | 424 mg/dose (100 μmol sulforaphane equiv.) | 20 | 11 | 0.55 |

The development of palpable tumors was delayed for as much as 5 weeks by the administration of sprout extracts. Rats treated with either Preparation A or B had significantly fewer tumors than the untreated control, and the multiplicity of tumors (tumors per rat) was significantly lower in the animals receiving Preparations A or B.

### *Example 11*

### *METABOLISM AND CLEARANCE OF GLUCOSINOLATES IN HUMANS*

Two male, non-smoking volunteers ages 35 and 40 years, each in good health, were put on a low vegetable diet in which no green or yellow vegetables, or condiments, mustard, horseradish, tomatoes or papayas were consumed. After 24 hours on such a diet, all urine was collected in 8 hr aliquots. After 24 hours of baseline data, subjects ingested 100 ml of broccoli sprout soup (prepared as below), containing 520 $\mu$mol of glucosinolates.

The sprouts were prepared by first surface sterilizing seeds of *Brassica oleracea* variety *italica* (broccoli) cultivar Saga with a 1 min treatment in 70% ethanol, followed by 15 min in 1.3% sodium hypochlorite with ca. 0.001% Alconox detergent. Seeds were grown in sterile plastic containers at a density of approximately 8 seeds/cm$^2$ for 72 hours on a 0.7% agar support that did not contain added nutrients. The environment was carefully controlled with broad spectrum fluorescent lighting, humidity and temperature control (16 hours light, 25°C / 8 hours dark, 20°C). The plants were rapidly and gently collected from the surface of the agar to minimize glucosinolate hydrolysis by endogenous myrosinase released upon plant wounding. A large quantity of sprouts was harvested by immediately and rapidly plunged into approximately 3 volumes of boiling water in order to inactivate endogenous myrosinase as

well as to extract glucosinolates and isothiocyanates from the plant tissue. Water was returned to a boil and maintained at a rolling boil for 3 min. Following the boiling step, sprouts were homogenized directly in their infusion water for 1 min using a Brinkman Polytron Homogenizer and the preparations were frozen at -79°C until use.

Inducer potential of plant extracts, prepared as described above, was determined by the microtiter plate bioassay method as described above. Inducer potential is nearly all due to glucosinolates; predominantly glucoraphanin, which is the glucosinolate of sulforaphane, but some glucoerucin which is the sulfide analog of glucoraphanin was also present. When converted to isothiocyanates by the addition of purified myrosinase, Phase 2 enzyme-inducing potential was 100,000 units/ml and contained 5.2 μmol of isothiocyanates per ml, as determined by the cyclocondensation reaction described in Example 7. Thus, the subjects consumed a total of 520 μmol of glucosinolates.

Collection of 8 hour urine samples was continued for an additional 30 hours. Urinary excretion of isothiocyanate conjugates (dithiocarbamates) was monitored using the cyclocondensation reaction as described in Example 7.

*TABLE 5*

*EXCRETION OF DITHIOCARBAMATES BY TWO SUBJECTS INGESTING 520 MICROMOLES OF GLUCOSINOLATES EXTRACTED FROM SAGA BROCCOLI*

| TIME | CONDITION | SUBJECT 1 | SUBJECT 2 |
|---|---|---|---|
| Collection Time (hours) | | μmol Dithiocarbamate per 8 hour urine collection | |
| 8 | baseline | 1.4 | 2.7 |
| 16 | baseline | 2.1 | 0.9 |
| 24 | baseline | 1.7 | 5.4 |
| 32 | 1st 8 hour post-dose | 23.2 | 20.4 |
| 40 | 2nd 8 hour post-dose | 9.9 | 36.8 |
| 48 | 3rd 8 hour post-dose | 4.4 | 14.0 |
| 56 | 4th 8 hour post-dose | 4.2 | 4.1 |
| Total post-dose minus average baseline: | | 39.8 | 63.2 |
| Total as Percent of dose: | | 6.7% | 12.2% |

The two subjects studied metabolically converted a significant fraction of the ingested glucosinolates to the isothiocyanates which were converted to cognate dithiocarbamates and measured in the urine.

*Example 12*

*EFFECTS OF PHYSICAL INTERVENTIONS ON SPROUT GROWTH ON PRODUCTION OF INDUCERS OF QUINONE REDUCTASE*

Sprouts were prepared by first surface sterilizing seeds of *Raphanus sativum* (daikon) by a 1 minute treatment with 70% ethanol, followed by a 15 min treatment with 1.3% sodium hypochlorite with approximately 0.001% Alconox detergent. Seeds were grown

in sterile plastic containers at a density of approximately 8 seeds/cm$^2$ for 7 days on a 0.7% agar support that did not contain added nutrients. The environment was carefully controlled with broad spectrum fluorescent lighting, humidity and temperature control (16 hours light 25°C/8 hours dark, 20°C).

Treated sprouts were irradiated with germicidal UV light for 0.5 hr on days 5 and 6. Treated sprouts were only half the height of the untreated controls. Plants were harvested on day 7 by rapidly and gently collecting the plants from the surface of the agar to minimize glucosinolate hydrolysis by endogenous myrosinase released upon plant wounding. Sprouts were harvested by immediate and rapid plunging into approximately 10 volumes of DMF/ACN/DMSO (1:1:1) at approximately -50°C in order to inactivate endogenous myrosinase as well as to extract glucosinolates and isothiocyanates. Sprouts were immediately homogenized with a ground glass mortar and pestle and stored at -20°C.

Inducer potential of plant extracts, prepared as described above, was determined by the microtiter plate bioassay method as described above. Inducer potential of the UV-treated sprouts was over three times that of untreated controls. Treatment of sprouts with ultraviolet light therefore increased the Phase 2 enzyme-inducer potential of the plant tissue.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention, which is defined by the following claims. All publications and patent applications mentioned in this specification are indicative of the

level of skill of those in the art to which the invention pertains.

All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference in its entirety.